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Interferometeric measuring of volume changes of photosynthesizing and non-photosynthesizing samples

Bachelor thesis

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Annotation: Possible applications of the interferometer principle to measuring of light induced volume changes of liquid and solid samples both *in vitro* and *in vivo* are treated. The description of devices which facilitate the measuring of photosynthesising samples by the interference of coherent beams is comprised, also with brief information about the processing of an interferometric signal. Few particular results are discussed, namely the light induced expansion of sheets of coloured papers, ink solution, suspension of chloroplasts or photosynthesizing bacteria, and bean leaves.

Prohlašuji, že svoji bakalářskou práci jsem vypracoval samostatně pouze s použitím pramenů a literatury uvedené v seznamu citované literatury.

Prohlašuji, že v souladu s § 47b zákona č.111/1998 SB. v platném znění souhlasím se zveřejněním své bakalářské práce, a to v nezkrácené podobě elektronickou cestou ve veřejně přístupné části databáze STAG provozované Jihočeskou univerzitou v Českých Budějovicích a na jejích internetových stránkách.

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Podpis:

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Contents

List of abbreviations used in the text:

[unit] *e.g.*: [s]	physical units
E	intensity of the electric field (chapter 3.) or energy (chapter 2.)
j	flow of energy
Δ	increment of a quantity
$\dot{z}$, $\dot{x}$, $\dot{A}$ and so on	time derivatives
μ	micro
n	nano
λ	wavelength of light
ω	circular frequency
π	Ludolph's constant: 3.14159…
φ	phase constant
t	time
A	amplitude
sin, cos	mathematical functions sinus and cosinus
s	second
min	minute
(number.) *e.g.*: (4.2.2.)	reference to the corresponding part of this text
(number, number) *e.g.*: (-1,1)	interval in mathematical sense
PAM	pulse amplitude modulation
Chl	chlorophyll

1. Introduction

Photosynthesis belongs to ones of the oldest photophysical and biochemical processes on the Earth. A large progress in the research of photosynthesizing organisms was achieved at the end of 20th century by introducing of modern optical methods and techniques. Among all, the chlorophyll fluorescence technique and photoacoustic spectroscopy appeared to be powerful tools for the studying of photochemical and non-photochemical processes within thylakoid membranes, chloroplasts, plant tissue, and whole plants (Schreiber *et. al* 1988, Bushmann *et. al* 1990). In this work, a new concept for investigation of photosynthetic phenomena is applied, namely the interference of beams of coherent light.

The main aim of this work is to study possible application of interferometer in plant physiology, specifically the study of the thermal dissipation of absorbed light during the photosynthesis. There have been no articles published on the topic of interferometry in the plant physiology yet (except Barabash *et. al* 2002, in which authors employed the holographic interferometry to the measurements of refraction index changes in reaction centra of *Rhodobacter sphaeroides*, which differs from this work), so there are not many references cited in this work. Therefore, everything written without quotation marks is my own opinion or conclusion and possible mistakes are on my own responsibility.

The scope of this work is to find possible ways for a proper application of the interferometric principle in a field of the photosynthetic research leading to the development of an instrument useful in the study of photosynthesis. From an early beginning, I participated in a construction of a new apparatus based on the Michelson interferometer and equipped with rather simple accessories. The first functional prototype was assembled in the Institute of Plant Molecular Biology, Biological Center of the Academy of Sciences of the Czech Republic (IPMB, BC AS CR, p.r.i.), in December 2004. We improved and tested the prototype during 2005 and 2006. In this work, the necessary apparatus improvements as well as measurements on both photosynthetically inactive and active samples, realized by me, are presented.

It is always difficult to start something new, so the main parts of this work are the “Methods” rather than the “Results”. This is also the reason, why the theory of an interferometer is included in ”Methods” and not in the chapter “Theory”. This makes the chapter “Methods” a compact description of the interferometry of photosynthetically active samples. There is important to say that the interferometer is well known device (*e.g.* Hariharan 2003, Hariharan 2007) but I was not able to employ all of available improvements, because I had to deal with the basic equipment (HeNe laser, mirrors and lenses). On the one hand, this can make the “Methods” a little bit outdated. On the other hand, it demonstrates that even simple interferometer can produce good results in rather "poor" conditions, in comparison with the already established photoacoustic method (Bushmann *at. al* 1990), shortly described in 2.3. that needs more sophisticated devices for basic measurements.

On the basis of the search in the *ISI Web of Knowledge* databases (http://isi17.isiknowledge.com/portal.cgi/), as mentioned, there have been no results published yet on the topic of application of interferometry to investigation of the primary photosynthetic processes. As demonstrated in literature, interferometric (often holographic) techniques were applied to determine inner cell structure after exposure to salt, low temperatures, ice grains thawing (Ning et al. 1994), to measure a thickness of the human cornea *in vivo* (Hitzenberger 1992), metioned paper of Barabash, *etc*.

2. Theory

2.1. Distribution of the excitation energy

Simply said, plants absorb low entropy light energy and transfer it to the high entropy substances to obtain work or any low entropy products. If we consider it more in detail we can draw this schema (Fig. 1):

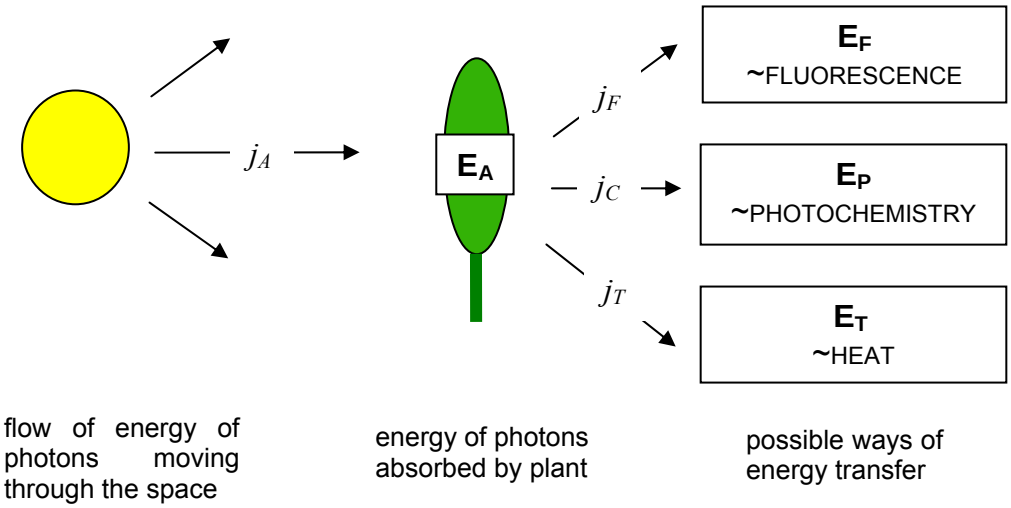

Fig. 1.: Scheme of distribution of the energy absorbed by a plant (E_A) in three main competitive pathways called fluorescence (E_F), photochemistry (E_P), and thermal dissipation (E_T). Corresponding energy flows are labeled as j_x.

Because the whole amount of absorbed energy must be conservative we can write for the energy increments:

$$\Delta E_A = \Delta E_F + \Delta E_P + \Delta E_T \qquad \text{(Eq. 1)}$$

or for the corresponding energy flows similarly:

$$j_A = j_F + j_P + j_T \quad \left(j_x = \frac{\partial E_x}{\partial t} \right) \qquad \text{(Eq. 2)}$$

(The meaning of the variables in Eq. 1 and 2 follows from Fig.1.)

The most exact way how to write Eq. 2 is to use a form of the equation of continuity which comprises the possible conservation of energy (*e.g.* in the light harvesting complexes) missing in Eq. 1 and 2:

$$j_F + j_P + j_T - j_A - \frac{\partial E_A}{\partial t} = 0 \qquad \text{(Eq. 3)}$$

(E_A means the absorbed energy conserved in plant and not dissipated or used yet).

2.2. Quantification of energy utilized by plant

If we study biochemical details of photosynthesis, we are most interested in energy consumed in biochemical processes E_P or its flow j_P, but it can not be measured *in vivo* with good time resolution (we can estimate it from the growing speed for example, but it is not suitable if we want precise information every minute or second). Nevertheless, we can measure this quantity indirectly. Using Eq. 3 we can simply write:

$$j_P = \frac{\partial E_A}{\partial t} + j_A - j_F - j_T \qquad \text{(Eq. 4)}$$

If we know variables on the right side of Eq. 4 then we know j_P. Flow j_A of energy into the plant is under our control because it is dependent on the source of light and we can adjust it according to the purpose. Method based on the measuring of fluorescence enables us to measure both j_F and partly E_A. j_F is simply the radiation released from the sample, which can be distinguished from the other radiation (like radiation responsible for the flow j_A). Part of the energy E_A conserved as the excitation energy used for a charge separation in reaction centra of the photosystem II can be also measured applying the technique of saturation flashes. More details on fluorescence measurement are beyond the scope of this text. It is important that if we could measure directly also the j_T we would obtain exact information about j_P.

2.3. Energy dissipated as heat

There exist a few methods how to measure energy dissipated as heat. The simplest one is to measure the surface temperature. If we want to measure temperature we need to use a proper thermometer. Even the tiniest thermometers (semiconductor-type probes or any other thermoelectric devices) have significant heat capacity and too slow response for biochemical purposes because of their buffering effect during fast photochemical processes. Thermometer needs a time to reach the thermal balance with the sample. The other important problem is that temperature is a quantity defined for macroscopic objects and it is difficult to define it out of equilibrium. A photosynthetically active sample is not at equilibrium and its surface temperature does not have to be simply correlated with the processes acting inside.

Although in measuring of spectra (*e.g.* absorption), we can use that some spectral properties of the sample (*e.g.* absorption maximum of water) are slightly dependent on temperature. But, it is almost impossible to measure those spectra on living plants. Spectral measurement needs intensive irradiation of the sample. This makes this method unsuitable for study of photosynthesizing samples.

One possible way how to measure energy dissipated as heat within the sample is to measure changes in the sample volume (sample thermal-induced dilatation). There were done attempts to measure it by the photoacoustic methods. Main idea of photoacoustics is to measure acoustic waves generated within the sample as a result of a light absorption. These measurements are complicated and need expensive and elaborated devices (Bushmann *at. al* 1990).

In our approach applying the interferometry, we can theoretically measure even tiniest movements of the sample surface (changes in order of hundreds of nm, may

be less) and so we can expect more precise results than those obtained by photoacoustic. There are also many problems, *e.g.*: released heat can be not the only one reason of expansion. Pressure of inner/outer gasses can change rapidly during physiological and photosynthetical processes, changes in turgor of cells can occur as well.

Despite of it, the interference technique appears to be the appropriate method for a recording of effects of the released heat. If this method is at least technically possible to be realized in a laboratory conditions and if the interferometry is able to compete with the photoacoustic method in the study of photosynthetically active samples is treated in this text.

3. Methods

3.1. Interference

Two linear coherent beams of light can be described as the two electromagnetic waves:

$$E_1 = A_1 \cos(\omega t + \varphi_1),\ E_2 = A_2 \cos(\omega t + \varphi_2) \qquad \text{(Eq. 5)}$$

$E_{1,2}$ mean the effective values of the respective vectors of the electric field intensity, $A_{1,2}$ are amplitudes of waves, ω is the circular frequency of a wave, t is time and $\varphi_{1,2}$ are phase constants of waves. (The consideration of all aspects connected with the fact that $E_{1,2}$ are vectors comprises effects like polarization, which can decrease the resolution and contrast of image on the screen, but they are not important for the basic understanding of the light interference phenomenon.)

Intensities of both beams are simply corresponding to A_1^2 respectively A_2^2, because the light intensity I is proportional to E^2 (Feynman *et. al* 2000). If the light beams meet each other at the same place their final intensity will be:

$$I = A_1^2 + A_2^2 + 2A_1A_2\cos(\varphi_1 - \varphi_2). \qquad \text{(Eq. 6)}$$

When $A_1 = A_2 = A$ we can simplify Eq. 6 using the (following) goniometric expression:

$$\cos^2\left(\frac{\alpha}{2}\right) = \frac{1+\cos(\alpha)}{2} \qquad \text{(Eq. 7)}$$

and finally conclude:

$$I = 4A^2\cos^2\left(\frac{\varphi_1 - \varphi_2}{2}\right). \qquad \text{(Eq. 8)}$$

The phase difference $\varphi = \varphi_1 - \varphi_2$ in Eq. 8 corresponds to the path difference ΔL of both beams and can be described using the following relation:

$$\varphi = \frac{2\pi}{\lambda}\Delta L, \qquad \text{(Eq. 9)}$$

where λ is the wavelength of the light used in the experiment.

3.2. Interferometer

Interferometer is a device, which uses the interference phenomenon for a determination of lengths and distances. Scheme of this device is in Fig. 2, (for more details see appendix or *e.g.* Hariharan 2003):

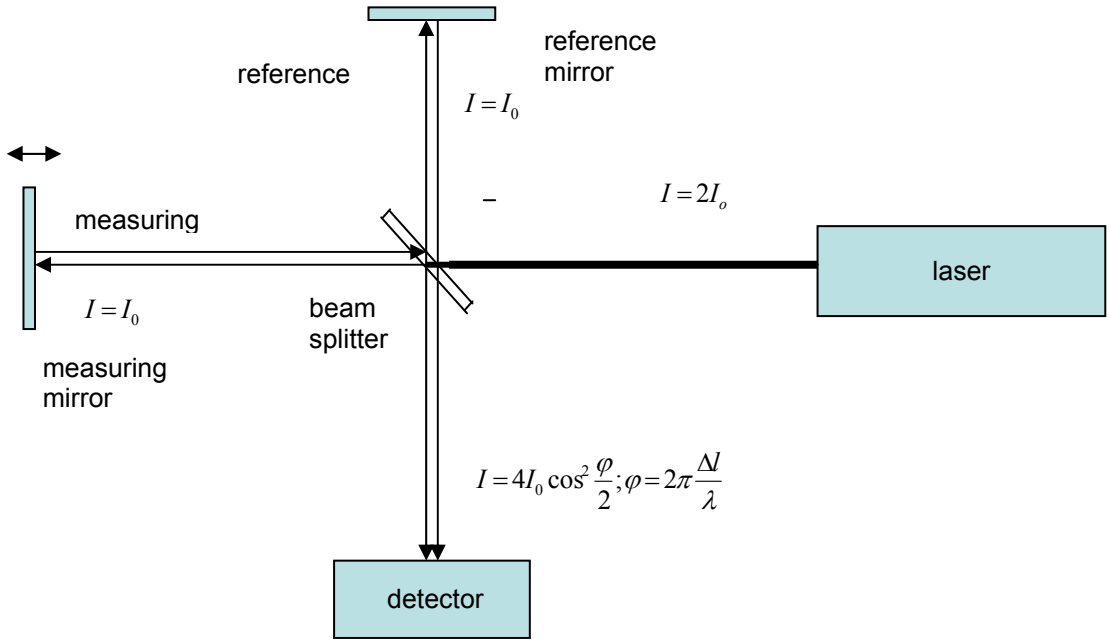

Fig. 2.: Basic scheme of the Michelson interferometer with a laser as a source of the coherent light.

We can derive from the Eq. 8 and 9 that if the reference mirror moves by the quarter of wavelength (corresponding $\Delta L = \lambda/2$) the light intensity on the screen will decrease from the maximum to minimum. This case is demonstrated in Fig. 3.

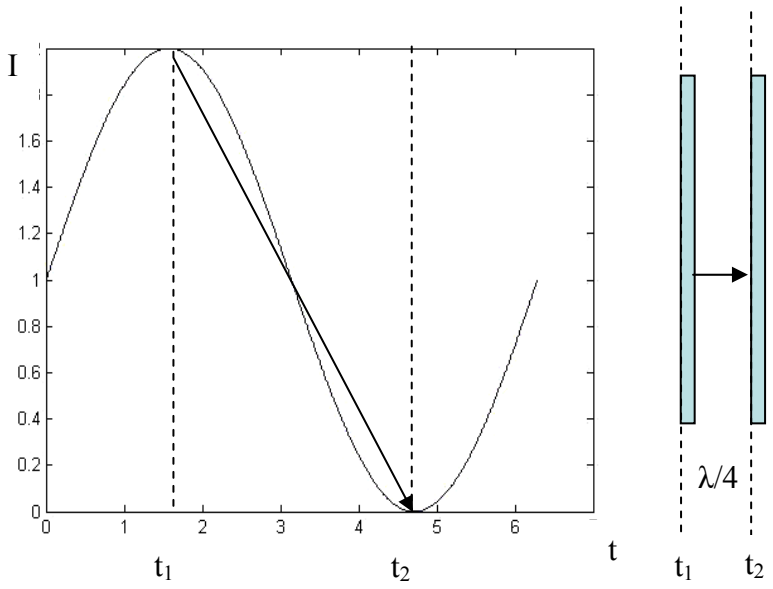

Fig. 3.: Distribution of the light intensity *I* in the time interval *t*, which corresponds to the regular movement of a reference mirror from 0 to the distance λ/4 (not λ/2 because measuring beam goes through its path twice: there and back). Both physical quantities are in relative units.

3.3. Adaptations of interferometer to the measurement of photosynthesizing samples

3.3.1. Interferogram recording and direction determination

Despite its construction simplicity, the interferometer is device that is difficult to apply properly. The theory described in 3.1. holds completely only when the both beams are perfectly coherent and collinear. In fact, the crooked circles or stripes of a certain width are seen on the screen as a result of the constructive or destructive interference. These stripes are running in the time in dependence on a velocity and direction of the reference mirror movement. Their time record obtained by means of a PIN-detector is called an interferogram. The width of a detector should be less than the width of the stripe to receive a good intensity resolution.

Interferograms consist of sets of intensity waves (see Eq. 8 and Fig. 5). To recognize the time-point at which the direction of the reference mirror movement is changed, I used two detectors situated horizontally on the screen in the distance of $\lambda/4$ between two neighbouring stripes (Fig. 4). This configuration enabled us to record both sin(Δφ) and cos(Δφ) like behaviour. It follows from Eq. 7 and the fact that $\cos(\varphi) = \sin(\varphi + \pi/2)$. The change-point in the direction of running stripes is detected by the "change in signals order" (see Fig.5).

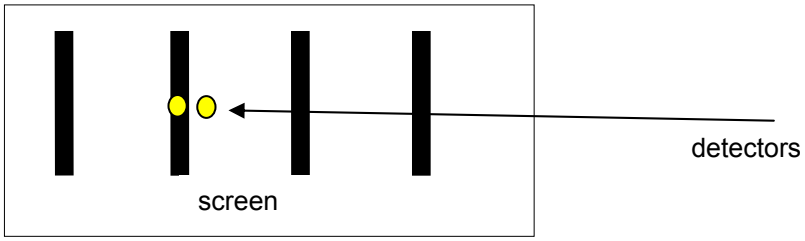

Fig. 4.: Configuration of two detectors in a screen plain for detection of change-points in the interferogram.

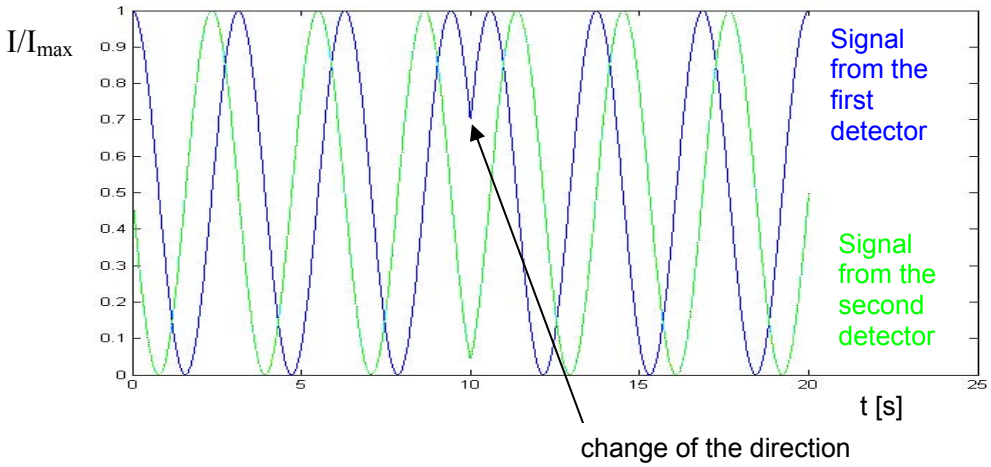

Fig. 5.: Two interferograms recorded at the same time using two detectors shifted one another by $\pi/4$. Initially, the "green" signal precedes the "blue" one. Ten seconds from the start of a record, the change-point arrived and the "green" signal started to follow the "blue" one.

3.3.2. Sample preparation and measurement

We measured interferograms on both liquid and solid samples and to this purpose we used completely different methods. First, we developed in collaboration with the Laboratory of Electron Spectroscopy (Institute of Entomology, Biology Centre AS CR, p.r.i.) small mirrors with a reflecting area of 4 x 4 mm^2 by means of the Au-layers deposition on thin glass chips. Using these small Au-mirrors, we were able to separate an intensive measuring laser beam from the own sample. This access allowed to avoid effects of a laser beam on the measured sample, because the used laser beam (HeNe laser, λ = 632.8 nm) was strong enough to overheat the sample material as well as damage the plant tissue or at least induce a strong photoinhibition of photosynthesis. Another reason was that the reflectivity of a leaf surface for a laser beam was too weak to receive a stable interferogram.

The most important thing was to connect the measuring Au-mirror with the sample surface so as to minimize side effects. It means to make the contact between the sample and the measuring mirror rigid but polite to the sample. Details are treated in special sections below.

For a sample irradiation, we used 150 W halogen lamp producing continuous white light and equipped with the light fibre-optics. The distance between the sample and the front end of the fibre-optics was adjusted to sample irradiation similar to the intensive daylight (1000 μmol/ m^2 s)

3.3.2.1. Liquid samples

I measured liquid samples in cuvette using a small floating mirror on their surface. If the mirror is enough thin it is held on the surface by capillary forces. Only problem is to make the mirror waterproof. We used mirrors prepared from microscope glass covered with a thin layer of gold. Often, the Au-layer was damaged by water penetrating under it. Because liquid surfaces are perfectly horizontal, there were no problem with the holding of the perpendicular position of the Au-mirror to the laser beam, but serious problems arose with the effect of water evaporation, which was verified to be very fast (at about 2 μm/s for a pure water) and capable to cover over the volume changes caused by absorption of an actinic light in a sample (approx. 1-2 μm/min). We also tested to measure liquid samples in closed cuvette by the use of the glass window made from the thin microscope glass (see Fig. 6). The glass was pasted to the cuvette before start of the experiment by a plastic adhesive material. It was important to find a material capable to paste a mirror fast, removably and air impermeably. Increasing pressure of evaporated water was able to cause an unpredictable leakage and thus influence the results. There was also problem with a water condensation on the inner side of the window. It dispersed the measuring beam and lowered the contrast on the screen. It was a problem of especially long-lasting measurements.

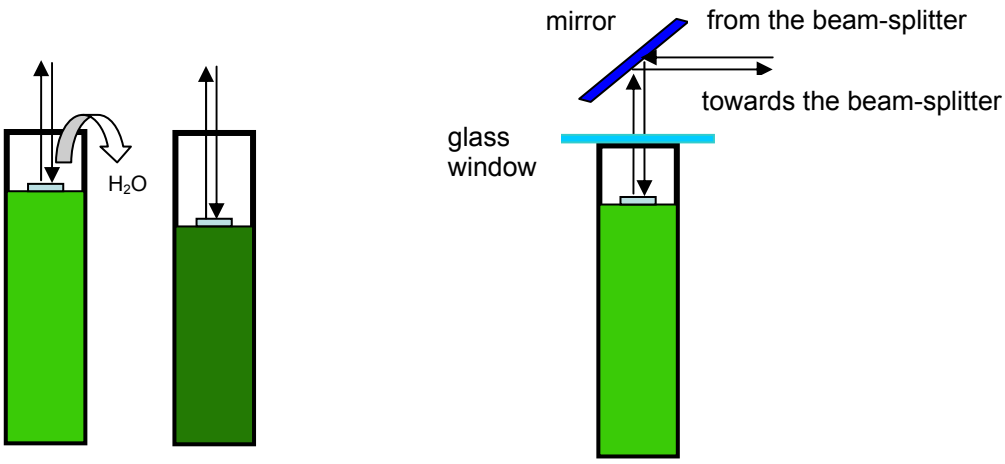

Fig.6.: The measuring principle for liquid samples. Liquid surface holds the Au-mirror in the horizontal position, but water evaporation from the sample moves the surface and influences results. This effect can be eliminated by covering the cuvette with thin glass plate.

3.3.2.2. Solid samples

Solid samples mean mass segments, *e.g.* whole leaves or parts of leaves or tissue, paper discs, *etc*. These samples usually have not a flat surface, so the problem is how to take hold the horizontal position of the Au-mirror if it is simply placed on the sample. It was solved by the use of a "transferring device". It is based on a pendulum principle and its scheme is shown in Fig. 7. The gravitation force holds the pendulum with the small Au-mirror in the horizontal position. Oscillations of the pendulum are eliminated with an adhesive matter (high viscosity lubrication matter in the pendulum joint). The transferring device was made by me of wooden skewers. This made the device structure light and practically not dependent on a laboratory temperature. Wood dilatation is mainly humidity-dependent but in laboratory conditions it is no problem to keep humidity stable during the experiment.

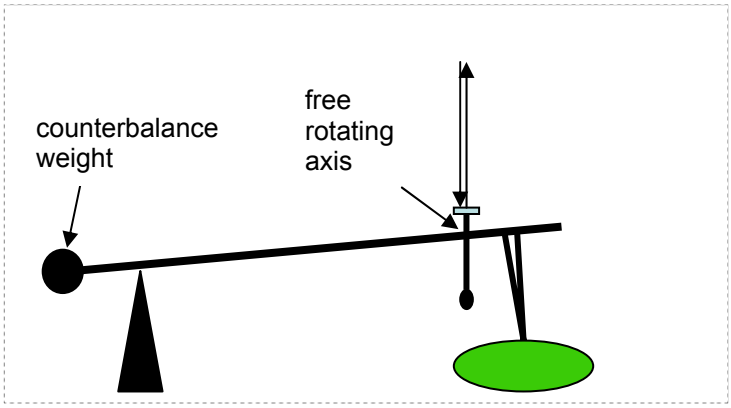

Fig. 7.: Scheme of the "transferring device" for measurements on solid samples.

Samples were fixed in a sample holder (developed by my supervisor K. Roháček), which enabled an influx of air to the photosynthetically active sample as well as illumination of a sample using fibre-optics. This device (schematically drawn in Fig. 8) was used for parallel measurements of the fluorescence emission and heat expansion of a planar sample. Opposite layers from a plastic foam fixed the sample but because of their flexibility they protected it from a pressure damage. It should be noticed that the probe of the transferring device was on the opposite site to the fibre-optics.

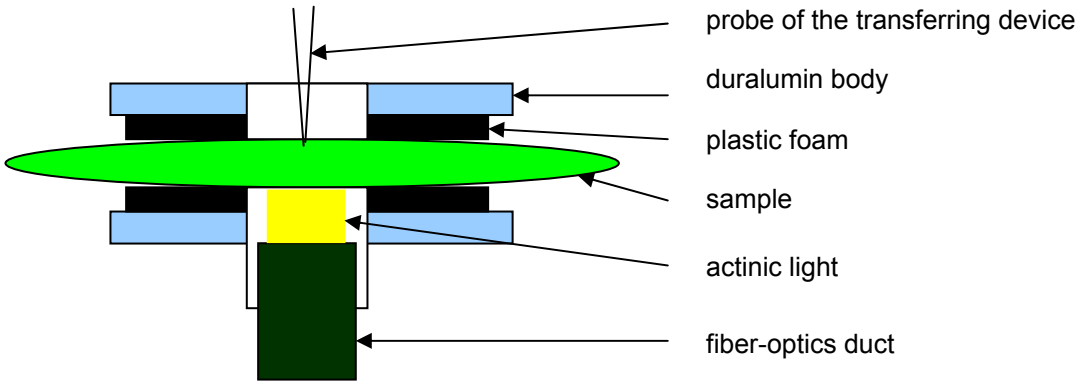

Fig. 8.: The sample holder for measurements on planar solid samples.

3.4. Signal processing

In my work, I checked a few methods of signal processing. Problems of interferometer signal processing were already solved (Hariharan 2003), but the device used by me had specific features, so I had to use less precise but more reliable methods. All methods described in this chapter were developed by me, but during experiments I found out that there are many works on this topic, in which it was already solved and described. Nevertheless, nothing was copied down from these works, so there are no references in this chapter. It will be important to study this literature thoroughly for the later development and application of the device.

3.4.1. Direct processing

Processing of the interferometric signal is a rather complicated problem. It is caused by the fact that we measure cos(φ) and cos is a 2π-periodic function. The inverse function arcos solves the problem only for the interval (-1, 1). It can be solved by using the two phase-shifted detector system (see 3.3.1.). I used two detectors that recorded signal so that the second detector was phase shifted by π/2 in comparison to the first one. In this case, we can write for the light intensities measured by the first and second detector ($I_{1,2}$) considering $A_1=A_2=A$ (see Eqs. 7-8):

$$I_1 = 2A^2(1+\cos\varphi) \qquad \text{(Eq. 10)}$$

$$I_2 = 2A^2(1+\cos(\varphi-\pi/2)) = 2A^2(1+\sin\varphi) \qquad \text{(Eq. 11)}$$

To make calculations more simple let us substitute $y_1 = \cos\varphi$ and $y_2 = \sin\varphi$. Notice that $y_{1,2} = \frac{I_{1,2}}{2A^2} - 1$, so if we can separate φ from $y_{1,2}$ we can obtain it from $I_{1,2}$, as well (they differ only by linear transformation). Let holds:

$$\dot{y}_1 = -\sin(\varphi)\cdot\dot{\varphi} \qquad \text{(Eq. 12)}$$

$$\dot{\varphi} = -\frac{\dot{y}_1}{y_2} \qquad \text{(Eq. 13)}$$

and finally:

$$\varphi = -\int_0^t \frac{\dot{y}_1}{y_2}. \qquad \text{(Eq. 14)}$$

This procedure works but it is very sensitive to the noise in the detected signal. It is because y_1 is a function numerically calculated from I_1 (Eq. 10), which was measured by the first detector. It is known that it is difficult to obtain applicable derivatives from numerical signal (Penrose 2007), especially when the signal is noisy or the sampling frequency is insufficient. In Eq. 13 the division by y_2 is applied and y_2 could be equal to zero in some cases. This fact makes this procedure almost inapplicable when the signal is noisy or the sampling rate is slow in comparison with the duration of one intensity period (the time, in which the following stripe reaches the position of the previous one. It could be solved by increasing the sampling rate but it is also important to hold the phase shift of detectors very precisely of π/2. This is very complicated in practice (but it can be solved by polarising components in interferometer but I had to make do without them), so I did not use this procedure finally.

3.4.2. Approximate processing

We can use a simple fact that the movement of the measuring mirror by λ/2 (and also the sample) causes exchange of two neighbouring signal maxima or minima on the screen (see Fig. 3). Thus, we can simply count number of waves passing over the detector and obtain the path change ΔL from it. It is not difficult to recognize maximum, minimum and the inflexion points of the sinusoidal function, so this method produces results with the resolution about $\lambda/8$. This method seems to be not very convenient but I had written the program in the MATLAB programming interpret (see the routine "mause" in Appendix), which uses clicks on the maxima, minima and inflexion points in the interferogram by a mouse to convert it in the path differences. In most cases, I used this method or some semi-automatic program (see 3.4.3. and routine "pocetvln" in Appendix) for a data processing.

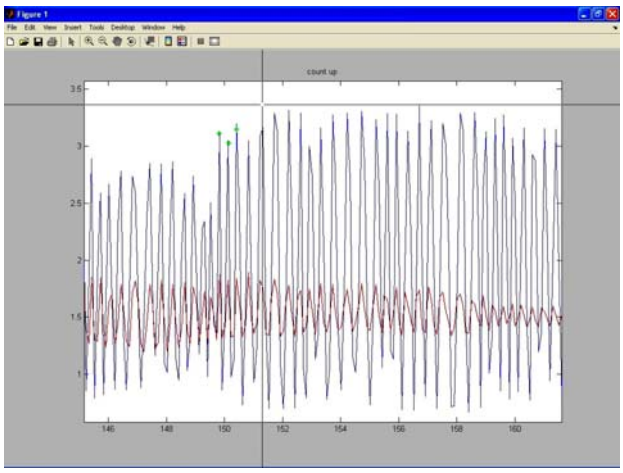

Fig. 9.: Example of an realistic interferogram and its processing. Black cross is the pointer of the mouse.

3.4.3. Semi-manual processing

The advantage of manual methods (see 3.4.2.) is that they are more reliable, especially when the signal is noisy and the phase shift between the two detectors is not exactly $\pi/2$. If the processing is made by the person who carried out the measurement he or she can use his own experience to distinguish signal from the noise (for example he or she can resolve disturbances occurring during measurements like a shaking of the device, opening the door, *etc.*). Of course, this method is not as fast as the fully automated procedures.

An ideal way to process the signal is the program that does the data conversion automatically and only in cases of the step uncertainty it asks the user for manual corrections (Fig. 9). The program in "user friendly" version is not completed yet but first version of this program is tested and works well (see the routine "pocetvln" in Appendix). It seems to be the best solution because it is impossible to develop both absolutely reliable and automatic signal processing program. It depends also on the device. Upgrading of the device components could increase a quality of the signal and the signal may become enough clear for an automatic processing.

4. Results

This chapter is divided in two main parts: the nonliving samples and the living samples. It is important to study nonliving objects. Studding the plants by measuring of its small dilatations is very indirect method. We have to know the moves of nonliving matter to distinguish, which moves display the processes connected with life. In fact, there is no strict line between these two. For example, dry leaf is nonliving object, but its reaction to the irradiation can tell us something about the living objects (they consist of the same matter). I tried to describe the way from the very physical objects (like ink or paper), across the objects like mentioned dry leaf or suspense of chloroplasts, to living object like a living plant *in vivo*.

4.1. Nonliving samples

4.1.1. Ink

One of the very first experiments was the measuring of the reaction of ink to the irradiation. The experiment design is explained in 3.3.2.1. Cuvette was tightly closed to make it air-impermeable. Light was switched on at the 300th second and turned off at the 600th second, see Fig 10:

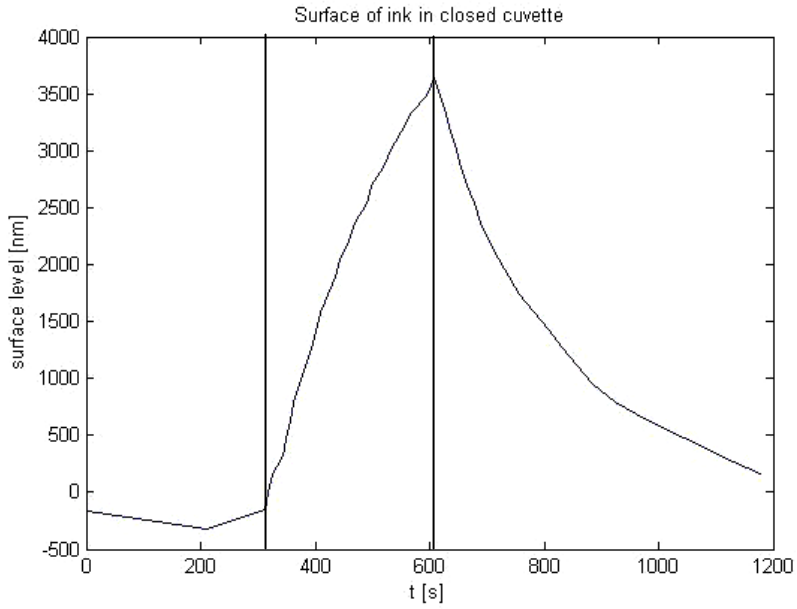

Fig.10.: Dilation of the Ink in cuvette as a reaction to the irradiation between 300th and 600th s.[1]

[1] It is important to say here that the light source was controlled manually, so if it is written: "light was switched off at the 800th second" it comprises small inaccuracy. It is sometimes noticeable in the graphs. Two vertical lines in each one of graphs in this chapter (4.) marks precise times of switching the light on respectively off.

Results were quite satisfying. Ink is a dead physical object and showed no complicated effects during the experiments. After the smoothly slowing expansion of the ink (during the irradiation) follows the smooth restoration of the initial state. If the changes of temperature are not vast, we can consider the thermal expansion linearly proportional to temperature, but the temperature itself changes not linearly, because the thermal conduction between the sample and its surrounding is rising with the increasing difference of temperature. It leads to the exponential dependence.
This result is quite promising for the later development of this method. If we measured living samples and found some peaks during the expansion or restoration, we would expect it had been an effect of a metabolism, because non-living samples shows no results like this. This not holds for the solid samples (4.1.2.).

4.1.2. Coloured papers

I used coloured papers to test if this method is able to distinguish light-induced expansion of absolutely same pieces of paper, differing only by their colour. Results were interesting but imply difficulty of this method. The method of the measurement is described in 3.3.2.2.
I measured black, green, yellow and white paper. They were from the same package and cut out to the same shape and size of 3 x 5 cm^2. Individual papers differed not only in the total reaction (It is clear that a black paper absorb much more light than a white, in the same time and under the same conditions) but also in dynamic. Amplitude of the paper moves is dependent on the measuring conditions, so the graph in Fig. 11 is in relative units to emphasis the shape, not the size of the curves. There are the curves displaying the level of the paper, dependent on time during irradiation in the Fig. 11:

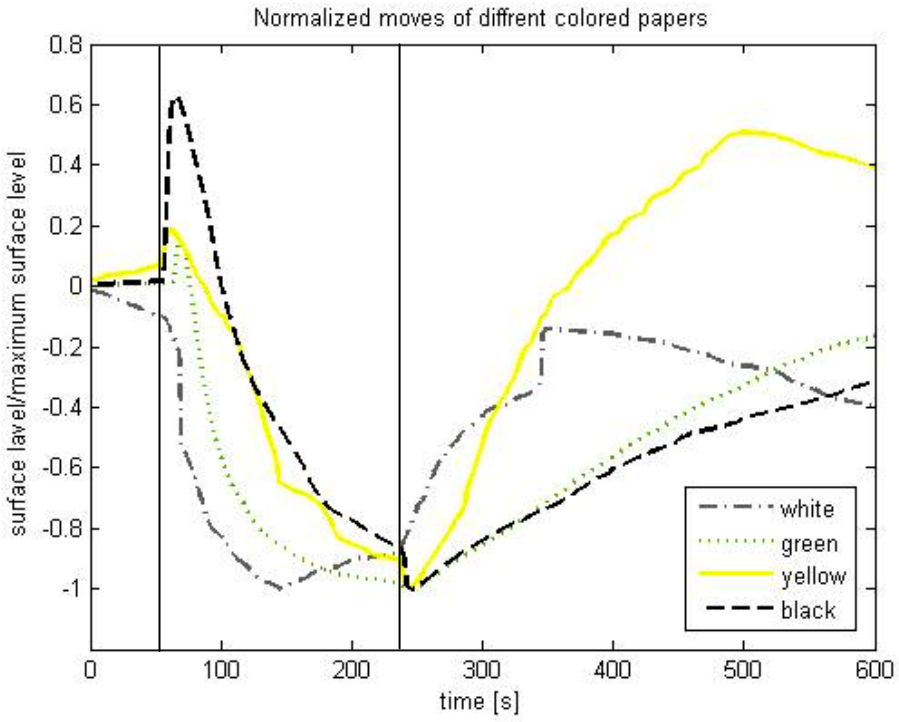

Fig. 11.: The surface moves of the same papers differing only in their colour, irradiated between 60th and 240th s.

It is little bit confusing, that the paper at the beginning starts to expand, and then rapidly changes its direction down. I suggest that it is a result of the combining of two effects: changes of volume and changes of shape. Paper expands and becomes thicker at first, and then bows down (see Fig. 12). Light is absorbed mostly in the middle of the paper, causing the expansion of the paper at first. Then the heat spreads along the sheet and consequently causes the bow as a result of a lateral dilatation. This theory explains why the surface of the paper moves up at first and then down. This effect is more intensive with dark paper because heating is faster in comparison with the heat conduction. If the heating is slow (white paper), both effects are in motion simultaneously. Later experiments showed large dependence of these effects to the type of the paper. Papers with the front side different from the underside tend to bend more of course.

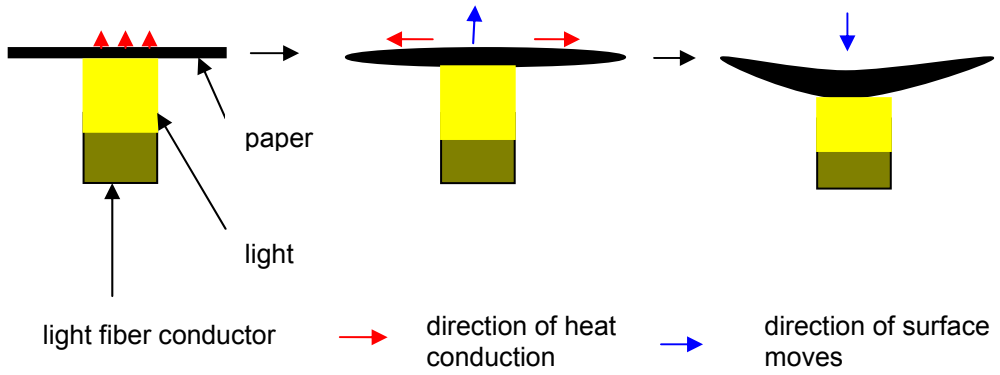

Fig. 12.: An explanation of the up-down movement of the sheet of paper (see Fig. 11) as a result of combining of consequent width and length dilatation. Holding device described in Fig. 8. is not depicted in this schema.

These effects are rather complicated and they can cause much confusion during the measurements of living samples. It can be partially solved by placing a transparent pad under the sample but it brings another problems. Pad can also absorb the light and expand a little, and gasses, released from the sample during the measuring, can move with the sample as well (see Fig. 13).

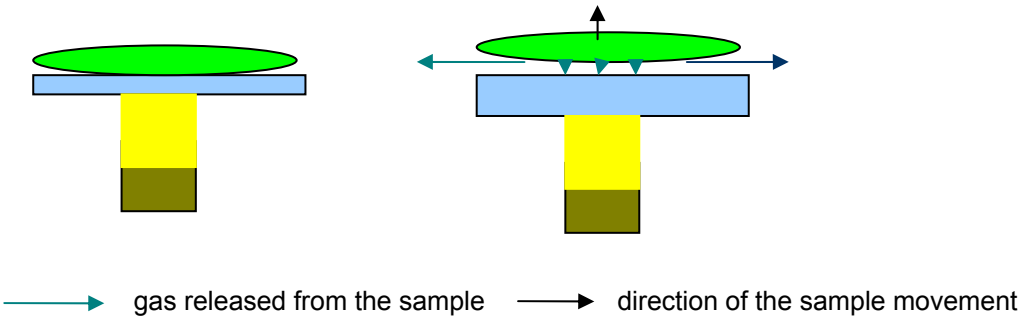

Fig. 13.: A placing of a pad under the sample can stop the bowing down but brings problems with released gasses and possible dilation of the pad.

Problems like this make this method somewhat ambiguous in obtaining of precise results on living samples now. It can be potentially solved by choosing of appropriate samples, or improving the method, like introducing some material for pad that is both gas and light transparent.

4.1.3. Dry leaf

Dried leaf is similar to the colored paper. Paper showed itself as a complicated subject in its behavior under light, so it is not surprising that the behavior of a dried leaf is even more complicated but similar to it, which is promising for the later interpretation of results. See the graph in Fig 14. The graph shows the surface of the dried leaf irradiated between 400th and 1200th s. The measuring method is described in 3.3.2.2. The curve is similar to results obtained on the green and yellow paper. Although the graph ends at the 1200th s, it can be noticed, that the restoration is not as fast as for the paper was. It can be explained by the expectation that the changes in volume of dried leave can be not wholly reversible (dried leaf is not flexible).

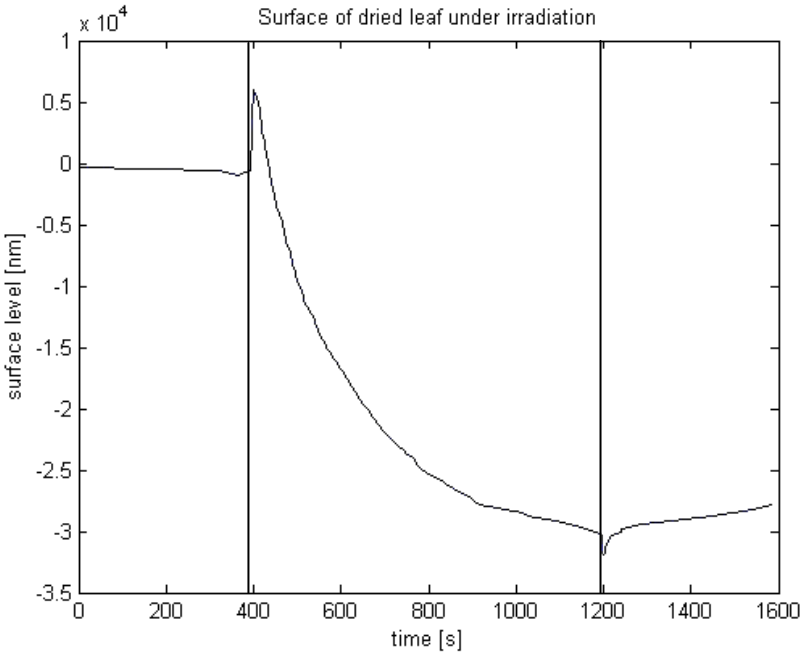

Fig. 14.: Surface of a dried bean leaf irradiated between the 400th and 1200th s.

It is not surprising. Structure of leave is much more elaborated and anisotropic than structure of paper. Biopolymers of the dried leaf can be fragile after their dehydration and so an intensive irradiation may cause irreversible damage to them. This also implies that the dried leaf does not have to be a good reference, if the living leaf is studied.

4.2. Living samples

4.2.1. Suspension of chloroplasts

The first measured living sample was the suspense of chloroplasts isolated from a pea (prepared by the courtesy of Ivana Hunalová). We did not repeated these experiment many times since it was on the very beginning of our experiments and we were interested especially in the technical side of the measurement. It was the first time we measured both thermal expansion (with the interferometer) and fluorescence (with the PAM-2000 fluorimeter) simultaneously, which was a goal of our experimental effort. The results are graphs in Fig. 15. The upper graph shows

the surface of the sample of chloroplasts during the experiment. The lover graph shows fluorescence of the sample. PAM fluorimeter served also as a light source. It was actually normal measuring of chloroplast fluorescence induction kinetics, only with the fact, that the thermal expansion was measured at the same time as well. The sample got the saturation flash at the time 100 s, actinic light started at the time 150 s and finished at the time 400 s. Note that saturation flashes shows no effect on the expansion. It is different from results obtained on the living leaf (4.2.3.2.). It is probably caused by water, which is a good thermal buffer.

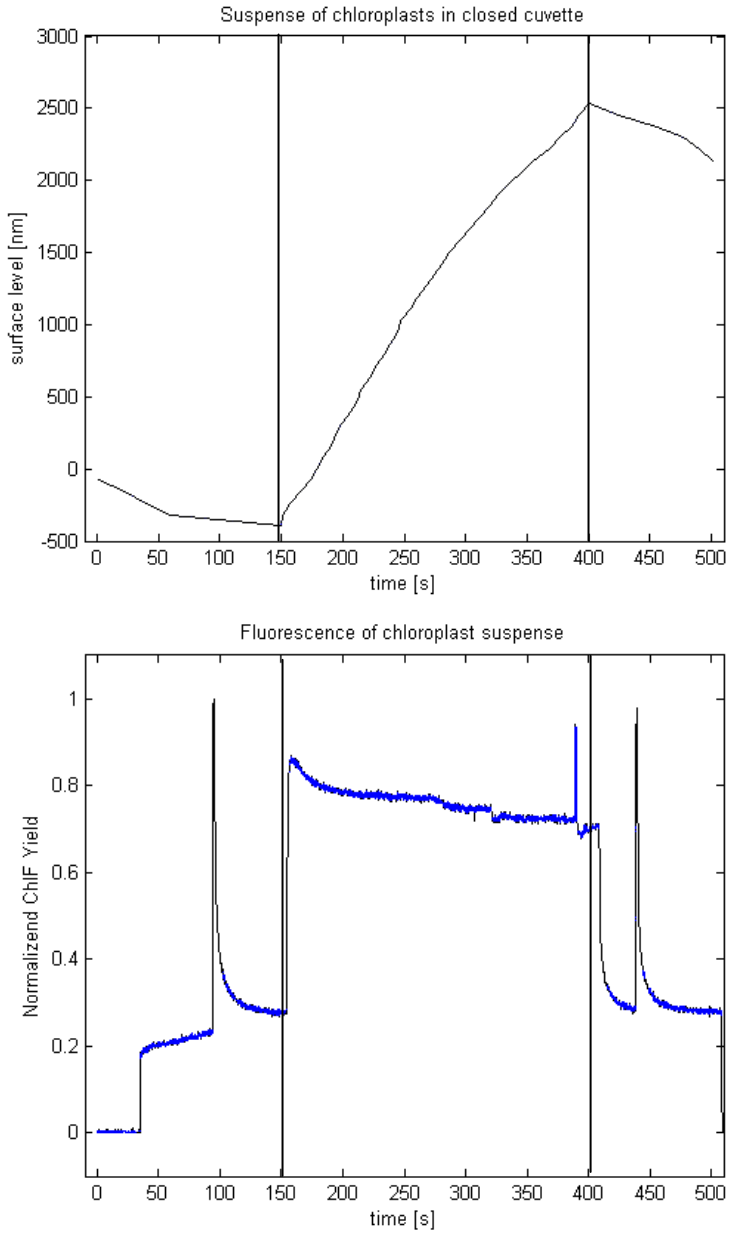

Fig. 15.: Dilatation and fluorescence of the solution of isolated pea chloroplasts during the induction of photosynthesis.

4.2.2. Solution of photosynthetic bacteria

We also tried to measure the thermal expansion of suspension of photosynthesizing bacteria (*Rhodobacter sphaeroides*). Method is described in 3.3.2.1. There are techniques to block their photosynthetic centra by specific chemicals (FCCP). I was interested if there would be differences between two samples, where the first do the photosynthesis and the second not. I expected that the latter would expand more, because more absorbed energy should be transferred to heat. Results were according the surmise. See the graph in Fig. 16. This graph shows the reaction of the suspension of photosynthetic bacteria to the irradiating (light between the 120th and 360th second). The measurement was carried out in a closed cuvette. It resembles the reaction of ink (4.1.1.) but there is a small difference between the sample with a chemically blocked photosynthesis and the normally photosynthesizing sample. The sample with blocked photosynthesis expanded more.

We also expected some difficulties in the curves during the induction of photosynthesis but in this case results were negative. Curves were similar to curves measured on the ink. Experiments were carried out both in the closed and opened cuvette (see Fig. 17). Results were in both cases similar to the previous ones (obtained in closed cuvette), but the measuring in opened cuvette showed more difficulties in its behavior. Problems with vaporizing are described in 3.3.2.1. I have not been able to explain the crossing of curves in Fig. 17. yet. This experiment should be repeated a few more times.

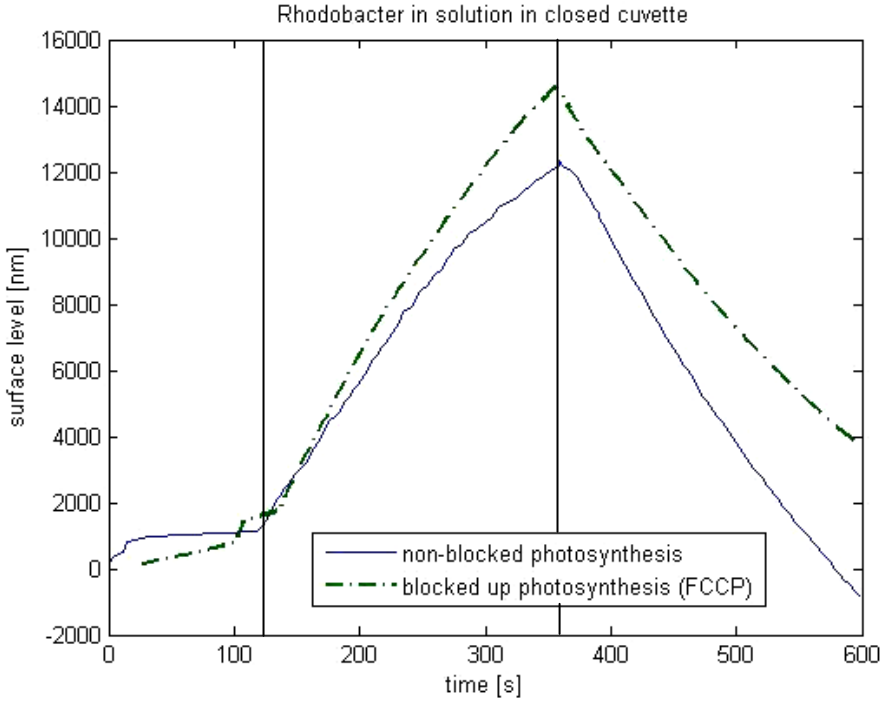

Fig. 16.: Light induced expansion of the solution of photosynthesizing bacteria with blocked and non-blocked photosynthesis, measured in a closed cuvette.

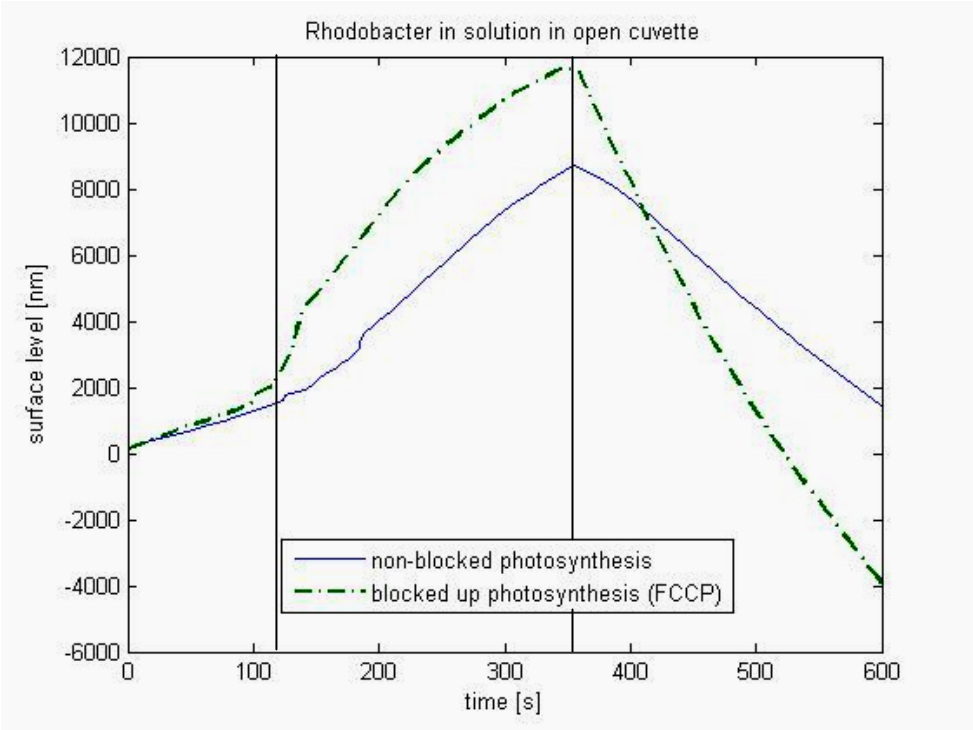

Fig. 17.: Light induced expansion of the solution of photosynthesizing bacteria with blocked and non-blocked photosynthesis, measured in opened cuvette.

4.2.3. Living leaf

I tried to measure both a living leaf-cut and a leaf directly on a living plant. These experiments imply that this method could confirm the results obtained by photoacoustic (Hamed 1998).

4.2.3.1. Living leaf cut off the plant

Firs experiment carried out on the living leaf was a measurement of the drying of the small circular cutting, cut off from the leaf of tobacco. I used method described in 3.3.2.2. These experiments showed a smooth slowing of the contraction. Process of the vaporization is difficult in general (Fanmann 2000) but in most cases we can expect something like the negative-exponential approaching of the final state and I measured this. Although we learned nothing more about the photosynthesis, this experiment showed us that the method using the transferring device described in 3.3.2.2. can bring in precise results when applied to the living leafs. See Fig. 18. A disc, about 1.5 cm in diameter wide, was cut off a tobacco leaf and left to dry few minutes and measured during it. The surface was approaching its final state by slightly slowing speed. Whole drying takes more than 24 hours so the graph shows only the beginning of this process.

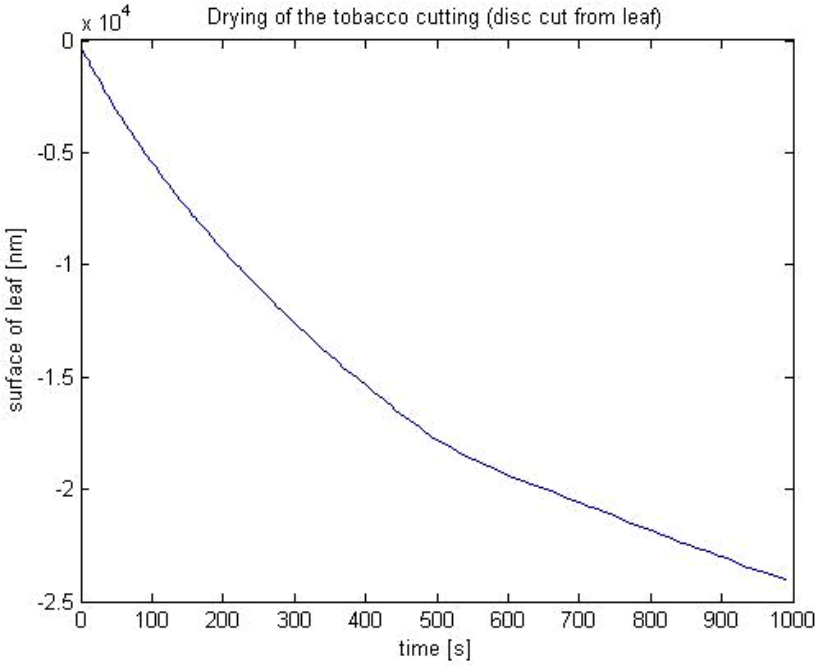

Fig. 18.: Contraction of the disc cut off a tobacco leaf, caused by its drying.

4.2.3.2. Leaves *in vivo*

I also tried to measure the bean leaf directly on the plant. Small plant grown in a flowerpot was brought to the device. Appropriate leaf (that means that this leaf had the suitable size and position on the plant) was fixed in the holding device described in 3.3.2.2. Only a small part of the plant was measured, while most of the plant was in dark adapted state, which could influence results. Leaf on the plant has a physiological hydrostatic potential so its behavior should be very realistic thou. Results were rather promising. This experiment was in agreement with the results obtained by much more complicated photoacoustic methods (Hamed 1998). When photosynthesis is induced in dark-adapted state, the sample should show expansion at firs (caused by the quick release of O_2) and then the contraction below the starting level (caused by the starting consumption of CO_2). In the end, when all processes reach the equilibrium, the sample should relax to the initial state. See Fig. 19. A dark-adapted leaf was irradiated and immediately measured, so the sample was under the light between the 0^{th} and the 800^{th} s. The firs peak, with maximum around the 3^{rd} min after the beginning of the experiment, may reflects the release of O_2, and the descent, with the minimum around the 9^{th} min after the beginning, can reflect the CO_2 consumption. The descent after the turning the light off can be caused by mechanic effects measured also on a paper and described in 4.1.2. There is no satisfying interpretation of the rapid expansion at the end of the experiment, but this effect appeared in repeated measurements, so it is not artifact. I have not found simple explanation of this phenomenon yet.

We also repeated this experiment with the measuring of both expansion and fluorescence of the leaf. We used the PAM fluorimeter as a source of light and measured fluorescence from the bottom side of the sample and the expansion from the upper side of the leaf (described in 3.3.2.2.).

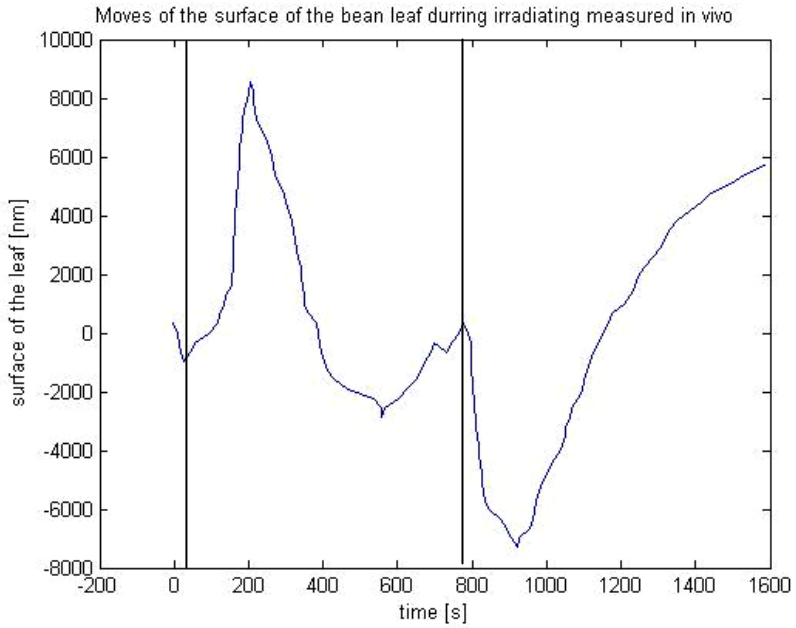

Fig. 19.: Dilation of a bean leaf during the induction of photosynthesis, measured *in vivo*.

Finally we obtained rather interpretable results, similar to those described above (Fig.19). See graphs in Fig. 20. These two graphs show the same experiment but from the different points of view. The upper graph shows expansion of the leaf and the lower graph shows its fluorescence. The leaf was measured directly on the living plant (*in vivo*). The sample was under the continual actinic light between the 3rd minute (180th s) and the 13.5th minute (810th s). The sample was also subjected to 6 saturation flashes that are evident in both graphs. We can see that saturation flashes can cause quite considerable expansion. It is different from the suspense of chloroplasts (4.2.1.). Note that in all cases sample returned back to the initial level after each saturation flash. The 3rd saturation flash is followed by switching the actinic light off, so the drop is little bit lower. This fast and little drop after the switching the light off was measured on the paper as well (4.1.2.). Similarly to the experiment above, the sample tended to expand at the beginning, and to decrease under the starting level as a probable result of O_2 production and subsequent CO_2 consumption. It continued to expand after the switching the light off. The chapter 4.1.2. describes, how difficult can the thermal expansion of a sheet of paper be, so there should be carefulness in this interpretation. Despite that, the results are satisfying. Further development can bring any new complications but also some improvement. I can not guess at this stage.

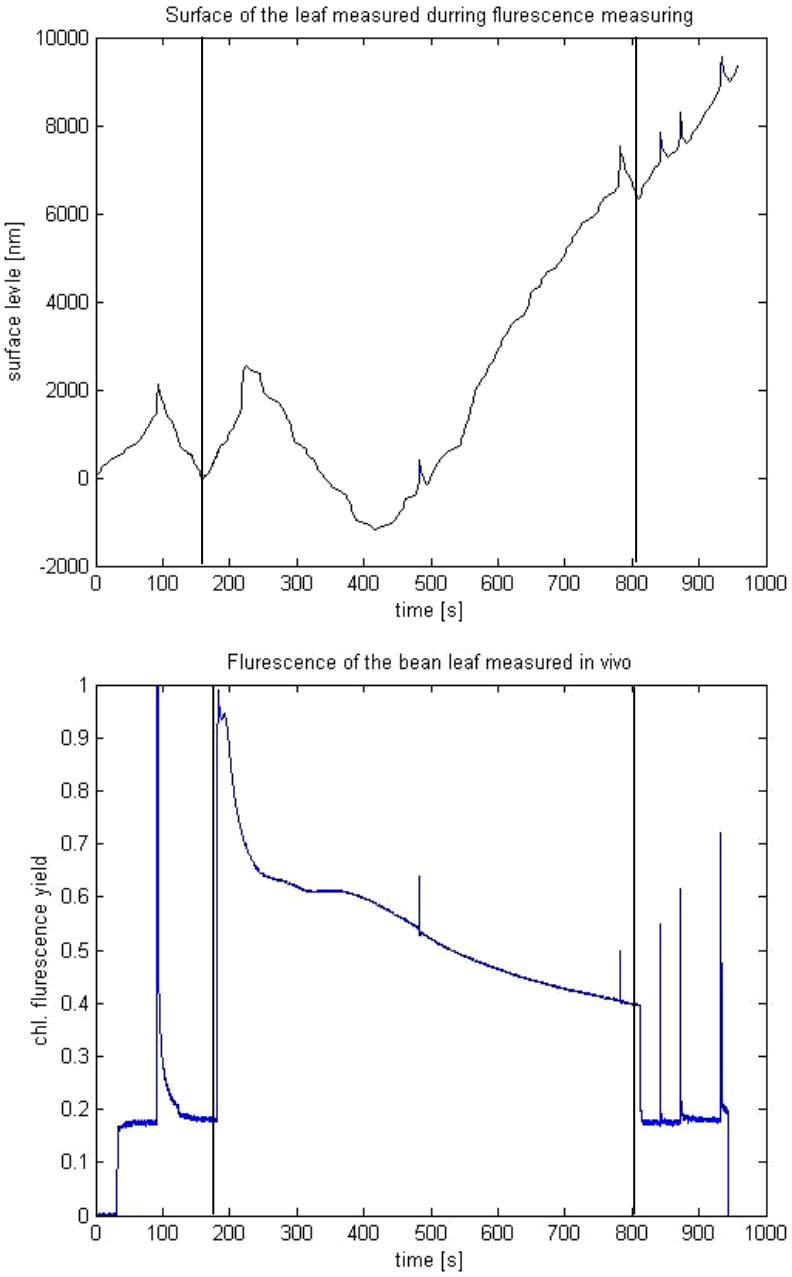

Fig. 20.: Dilation and fluorescence of a bean leaf measured simultaneously during the induction of photosynthesis, measured *in vivo*.

5. Discussion

There are several problems to discuss. The device was not able to reach the ideal sampling frequency and the most of our measuring were carried out by 10 or 20 records per second. Occasionally, especially during fast processes (it means that the sample surface moves faster than 2 μm/s), this frequency was insufficient. It can make some peaks in graphs a little bit higher or lower. This problem could be solved by an upgrading of the device.

It is important to say that although all results in this work originates from experiments which were successfully repeated, some of them need a more proper study. In some cases all measured samples were from the same source (plant) or measured in the same day, especially 4.2.1., 4.2.2., and 4.2.3.2.

Interpretations of all obtained results can show itself as wrong. They were not based on any other type of experiment. It is not satisfying, but this text is focused on the possible application of the interferometry and described interpretations are only attempts, trying to estimate possible problems in developing of constituent approaches, that need more profound investigation. The dynamics of movements during the irradiation is a convolution of many effects. On the other hand, living samples show indisputably more complex light induced dilatation behavior than the non-living samples, so the results obtained by this method really reflect some of life processes.

The study of more suitable samples can bring the deeper insight into the problem. It means mosses with one-cell thick leaves or some bacteria, which do photosynthesis without gas evolution *etc.*

Because of the problems with the sampling frequency, we have carried out no experiments to study vibration frequency spectra during the photosynthesis induction, which photoacoustics is often concerned about (Bushmann 1990). This approach may be promising for future experiments, although problem has been studied by photoacoustics yet, but it could not been tested with current prototype of the device.

6. Conclusions

Experiments described in this work showed that it is possible to study the expansion of photosynthesizing samples by means of an interferometer. It comprises solutions of photosynthetic bacteria (4.2.2.) or chloroplasts (4.2.1.), parts of leafs (4.2.3.1.) and whole leaves, also *in vivo* (4.2.3.2.). I developed facilities to enable it to us (3.3.2.). At now, we can obtain proper information about the sample surface moves during the induction of photosynthesis (4.).

From the technical point of view there are serious problems with the preservation of the perpendicular position of the measuring mirror (connected with the sample surface) to the measuring beam and with a noise background in the signal (because interferometers are very sensitive to vibrations). The former problem can be solved by special devices but the latter needs very still conditions, hardly reachable in the open air. There are also problems with unambiguous determination of the direction of sample movements (3.3.1.).

From the interferograms measured on the nonliving samples, we can derive, that expansion, caused by an irradiation, can show rather a complex behavior (4.1.2.), especially when planar objects, with complex inner structure, as sheets of paper or whole leaves, are measured. It can significantly influence the possible interpretation of results obtained by this technique, because these generally physical effects can outweigh photosynthesis-connected effects, and it seems not easy to eliminate them.

These effects are not significant in results recorded on liquid samples. Non-living liquid samples show very simple response to the irradiation (4.1.1.). On the other hand, liquid samples bring smaller chance to measure fast and tiny effect, because of the high heat “buffering” capacity of water. In this case the reaction of photosynthetic samples is very similar to reactions off non-photosynthesizing samples, but there are noticeable differences as well (4.2.2.). Despite mentioned problems I find liquid samples to be more suitable for the later development of interferometer-measured photochemical processes, because of much less difficulty in treating of non-photosynthetic effects.

In general, the interferometry can tell us quite proper information about the mechanical behavior of the large scale of samples. On the other hand interpretations of these results appear to be often ambiguous and largely dependent on the sample properties and measuring conditions.

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8. Appendix

8.1. Semi-manual MATLAB script for a data processing

The program requires function "mause" which is stated below. You use this function "pocetvln" according standard MATLAB rules as function called "pocetvln", which returns two vectors (x, y) and require parameters:
x…..independent variable (t [s] in most cases)
y1…signal from the first detector normalized between (-1, 1)
y2…signal form the second detector normalized between (-1, 1)
It is possible change parameter "treshold" directly in the program. It should have value between 0 (absolute noise tolerant) and 1 (no noise tolerant). Default value 0.7 is highly recommended.
Syntax is:

[x values, y values] = pocetvln(x, y1, y2);

At the beginning the program shows you a graph of data and you can mark points of changes of the direction by the left button of the mouse.

You operate program only by the mouse. From now "left" means a click by the left button and "right" means a click by the right button:

right, right … zoom area between these two points
(each click means one corner of zoomed rectangle)
right, left …….zoom out
left……pointing the place of direction change.

You return back by the click of the middle button.

```
function    [rx ry]=pocetvln(x,y1,y2)


[mousx mousz mousy]=mause (x,y1,y2);

[s1 s2]=size(y1);


[ms1 ms2]=size(mousx);
mus=1;

dir=1;

treshold=0.7;

e=0;
h=0;
prirustek=1;

for i=(1:s2);
    a=y1(1,i);
    if (dir==1);
    if (a>treshold);
        e=e+1;
        if(mus<=ms2)
        if (x(1,i)>mousx(1,mus))
            prirustek=-prirustek;
            mus=mus+1;
        end
        end
        h=h+prirustek;
        ry(1,e)=h;
        rx(1,e)=x(1,i);
        dir=-1;
```

```
    end
    end

    if (dir==-1);
    if (a<-treshold);
        e=e+1;
        if(mus<=ms2)
        if (x(1,i)>mousx(1,mus))
            prirustek=-prirustek;
            mus=mus+1;
        end
        end
        h=h+prirustek;
        ry(1,e)=h;
        rx(1,e)=x(1,i);
        dir=1;
    end
    end


end;

ry=ry./4.*632.8;

plot(rx,ry);
```

This program require function “mause” for manual processing:

```
function [k l n]=mause(x,y,y1);

hold off;
plot(x,y);
hold on;
plot(x,y1,'color','r');
axis([min(x-50) max(x+50) min(y-1) max(y+1)]);
i=1;
c=3;
w=0;
sign=1;
title('count up');

k(1,1)=0;
l(1,1)=0;

while (c ~= 2)
    [a b c]=ginput(1);
    if (c == 3)
        [p q r]=ginput(1);
        if (r == 3)
            if (a<p)
                axis([a p q b]);
            else
                sign=-sign;
                if (sign== -1)
                    title('count down');
                else
                    title('count up');
                end
            end
        else
            axis([min(x-50) max(x+50) min(y-1) max(y+1)]);
        end
    end

    if (c == 1)
        k(1,i)=a;
        l(1,i)=b;
        w=w+sign;
        n(1,i)=w;
        i=i+1;
    end

    if (k(1,1)~=0)
       plot(k,l,'*','color','g');
    end
end
k=sort(k);
hold off;
```

8.2. Schema of the device